Original Contribution

Benign coxsackievirus damages heart muscle in iron-loaded vitamin E-deficient mice

Melinda A. Beck[a],*, Qing Shi[a], Virginia C. Morris[b], Orville A. Levander[b]

[a]University of North Carolina, 3312 Medical Bio-Molecular Research Building, CB 7224, Chapel Hill, NC 27599-7224, USA
[b]Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, USA Department of Agriculture, ARS, Beltsville, MD 20705-2350, USA

Abstract

Several oxidative stressors (dietary selenium deficiency, dietary vitamin E deficiency coupled with fish oil feeding, genetic reduction of glutathione peroxidase activity) allow a normally benign coxsackievirus B3 (CVB3/0) to damage heart muscle in host mice. This study investigated whether dietary iron overload, another oxidant stress, would also permit CVB3/0 to exert a cardiopathologic effect in vitamin E-deficient ($-$VE) mice. Four groups of mice were fed either a $-$VE or a $+$VE diet containing either an adequate or an excessive ($30\times$) amount of iron. After 4 weeks of feeding, the mice were inoculated with CVB3/0 and heart damage was assessed at various times postinfection. Mice fed a diet sufficient in VE with excess iron developed heart damage equivalent to mice fed a diet deficient in vitamin E without excess iron. However, severe heart damage occurred in the group fed a diet deficient in VE with excess iron, which was the most pro-oxidative diet. The highest heart viral titers were found in mice fed the $-$VE/excessive iron diet. However, the extent of heart damage did not always correlate with the formation of TBARS in liver homogenates. Further research is needed to clarify the role of oxidative stress and iron overload in determining the course of viral infection.
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Introduction

The nutritional essentiality of iron has long been recognized and indeed iron deficiency anemia remains the most widespread nutritional deficiency in the world today [1]. Despite the body's obvious need for iron, it is also true that too much iron can have toxic effects in humans. Perhaps the most graphic example of iron's potential to harm is the fact that accidental consumption of iron supplements is a leading cause of poisoning death in children [2]. Deleterious effects of chronic iron over-exposure have also been suggested and some workers have proposed that dietary iron overload could play an etiological role in a variety of human ills such as cancer or heart disease [3,4].

The mechanism by which long-term consumption of excess iron exerts its harmful effects is not clear but could involve its ability to induce oxidative stress by generation of the hydroxyl free radical via the Fenton reaction. The high reactivity of the hydroxyl free radical makes this a potent pro-oxidant which can damage cellular lipids, proteins, and nucleic acids [5].

Previous work from our laboratories has shown that in vivo oxidative stress produced by feeding mice diets deficient in selenium or vitamin E stimulates conversion of an amyocarditic coxsackievirus to a myocarditic strain capable of causing heart muscle pathology [6,7]. In the case of the vitamin E-deficient mice, the cardiac damage induced by the coxsackievirus could be exacerbated by the concomitant feeding of fish oil, a known dietary antagonist of tocopherol [8]. It occurred to us that feeding other dietary pro-oxidants to vitamin E-deficient mice also might increase coxsackievirus-induced heart muscle damage as feeding fish oil did.

Excessive iron exposure is known to exert a pro-oxidant stress in vivo and considerable evidence has accumulated associating hepatic lipid peroxidation with chronic iron overload [9–12]. The purpose of the research reported here was to determine whether high dietary iron intake would allow a normally avirulent coxsackievirus to cause heart damage in either vitamin E-sufficient or -deficient mice.

Materials and methods

Mice

Three-week-old male weanling C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed four per cage with free access to the appropriate specified diet and water. All mice were housed in the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Review Board.

Diets

Four different diets were utilized in this study: two lard-based diets with vitamin E and two lard-based diets without vitamin E. The two E-deficient and two E-supplemented diets contained either 35 or 1050 μg Fe/g added as ferric citrate. The vitamin E-deficient diets consisted of the following (g/100 g): casein (20), tocopherol-stripped lard (4), tocopherol-stripped corn oil (1), AIN-76 salt mix without selenium (3.5), AIN-76A vitamin mix without vitamin E (1), DL-methionine (0.3), choline bitartrate (0.250), and sucrose (70). Selenium was added to all the diets at 0.2 μg/g as sodium selenite. For vitamin E-supplemented diets, vitamin E was added at 100 mg/kg as *RRR*-α-tocopheryl acetate. All dietary ingredients were purchased from Harlan Teklad (Madison, WI, USA) except for the DL-methionine, which was purchased from ICN (Irvine, CA, USA), and the sucrose (purchased locally).

Virus

The noncardiovirulent coxsackievirus B3 (CVB3/0) was propagated in HeLa cell monolayers in minimal essential medium supplemented with 10% fetal bovine serum and 50 mg/L gentamicin (GIBCO BRL, Gaithersburg, MD, USA) at $37°C$ in a humidified 5% CO_2 atmosphere. Virus was titered by tissue culture infectious dose-50 ($TCID_{50}$) [13].

Infection of mice

After consuming the specified diets for 4 weeks, mice were inoculated intraperitoneally with 10^5 $TCID_{50}$ of CVB3/0 in 0.1 ml of RPMI 1640 medium. Mice were killed by cervical dislocation at specified times after inoculation.

Determination of liver iron and α-tocopherol concentrations

Uninfected mice fed the same diets as the infected mice were used as index mice. Hepatic iron concentrations in index mice were determined by flame atomic absorption spectrometry as described by Hill et al. [14]. Livers from index mice were analyzed for vitamin E by a slight modification of the HPLC technique of Bieri et al. [15].

Histopathology

At 3, 5, 10, 14, and 21 days postinfection, mice were killed and their hearts removed for study. Hearts were rinsed in saline and cut transversely in half. One-half of each heart was immediately embedded in OCT (Optimal Cutting Temperature; Fisher Scientific, Pittsburgh, PA, USA) and frozen at $-80°C$. Frozen hearts were sectioned (6 μm) on a Zeiss HM 505 N cryostat (Carl Zeiss, Inc., Thornwood, NY, USA) and stained with hematoxylin and eosin. The extent of inflammatory lesions within the myocardium was graded by two independent pathologists, blinded as to experimental group, in a semiquantitative manner according to the relative degree (from heart to heart) of mononuclear cell infiltration and the extent of necrosis [16].

Heart viral titers

At 3, 5, 10, 14, and 21 days after inoculation, mice were killed and one-half of the heart was removed for viral titers. Heart halves were rinsed in saline and immediately frozen on dry ice and then stored at $-80°C$ until processed. Heart sections were weighed, then ground in a small volume of RPMI 1640 using a Ten Broeck homogenizer (Fisher Scientific), and freeze–thawed three times. The ground tissue was then centrifuged (2000*g*), and the resulting supernatant was recovered for assay. Supernatant was titered on HeLa cell monolayers by $TCID_{50}$ [13].

Measurement of thiobarbituric acid-reactive substances (TBARS) in liver

Hepatic TBARS were measured in 10% liver homogenates by the procedure of Jentzsch et al. [17].

Statistical analysis

The data were analyzed using two-way ANOVA and group means were compared using Tukey, Scheffe, or Bonferroni corrections for multiple comparisons where appropriate.

Results

The nutritional status of the mice used in our experiments with regard to iron and vitamin E was confirmed by

Table 1
Hepatic iron and vitamin E concentrations as influenced by dietary iron overload and vitamin E deficiency in uninfected mice

Diet		Liver Fe content (μg/g, wet wt)	Liver vitamin E content (μg/g, wet wt)
Fe (ppm)	Vitamin E (ppm)		
35	100	50 ± 2^a	40.5 ± 3.0^a
35	0	49 ± 3^a	1.1 ± 0.4^b
1050	100	126 ± 10^b	31.2 ± 3.7^a
1050	0	118 ± 5^b	1.3 ± 0.1^b

Mean values $\pm$ SD ($n = 5$). Means in the same column with different superscript letters differ significantly at the $p < 0.05$ level.

analyzing liver tissues from uninfected animals for their content of these nutrients. As expected, hepatic iron levels reflected the amount of iron added to the diet (Table 1). In the E-supplemented mice, increasing the dietary iron level by 30-fold led to a 152% increase in liver iron. Vitamin E status had no significant effect on hepatic iron levels. Infection with the coxsackievirus had no effect on liver iron content except in the vitamin E-supplemented mice fed a high level of dietary iron, in which case infection

caused an 18% drop in liver iron (data not shown). Mice fed the vitamin E-deficient diets had very low hepatic α-tocopherol contents compared to their corresponding vitamin E-supplemented controls (Table 1). The high iron level fed (1050 μg/g) tended to decrease liver vitamin E content in the vitamin E-supplemented mice, but otherwise dietary iron had no effect on the vitamin E content in the liver.

As we have reported earlier, mice fed a diet with normal levels of vitamin E and iron do not develop myocarditis when infected with CVB3/0 (Figs. 1 and 2), whereas mice fed a diet deficient in vitamin E develop a moderate level of myocarditis, which peaks at day 10 postinfection. Interestingly, the mice fed a diet sufficient in vitamin E but with high iron developed myocarditis equivalent to that of mice fed a diet deficient in vitamin E containing normal iron (Figs. 1 and 2). The most severe heart damage occurred in mice fed the vitamin E-deficient diet containing a high level of dietary iron.

In most groups, cardiac viral titers peaked at 5 days postinoculation (Table 2). In the vitamin E-deficient mice, viral titers were significantly elevated in mice fed the high level of dietary iron. Viral titers also tended to be higher in vitamin E-sufficient mice fed the high level of dietary iron. All dietary groups were able to eventually clear the virus. However, the mice fed the vitamin E- and Fe-sufficient diet cleared the virus at an earlier time point compared with the other diet groups.

At day 10 postinfection, for the normal level of dietary iron, TBARS in incubated liver homogenates were higher in infected mice fed vitamin E-deficient vs those fed the vitamin E-supplemented diets (Table 3). There was a trend
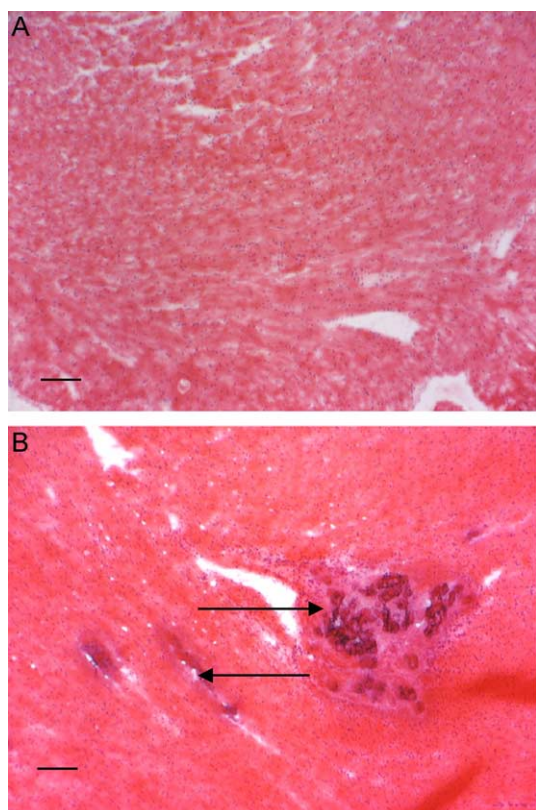

Fig. 1. Histopathology of left ventricular myocardium of mice inoculated with CVB3/0 10 days earlier fed a diet (A) supplemented with vitamin E and 35 μg ferric citrate/g or (B) vitamin E deficient with 1050 μg ferric citrate/g. Note the absence of inflammatory infiltrates in (A) compared with the large area of inflammation, necrosis, and dystrophic calcification seen in (B) (arrows). Heart sections were stained with hematoxylin and eosin. Bar in lower left-hand corner represents 100 μm.
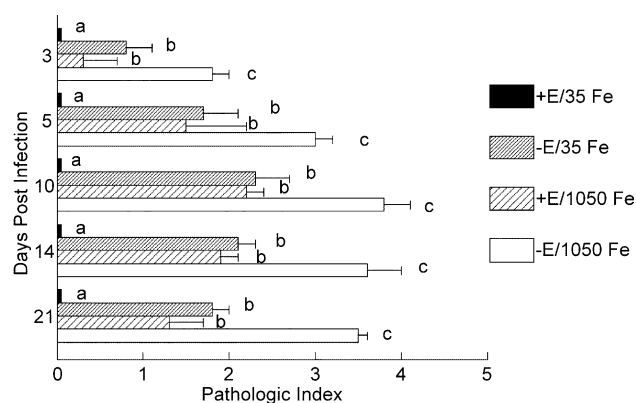

Fig. 2. Effect of diet on the development of myocarditis. Mice were fed the indicated diets for 4 weeks and then infected with CVB3/0. Heart muscle damage postinfection was assessed by assigning pathologic scores as follows: 0, no lesions; 1+, foci of mononuclear cell inflammation associated with myocardial cell reactive changes without myocardial cell necrosis; 2+, inflammatory foci clearly associated with myocardial cell reactive changes; 3+, inflammatory foci clearly associated with myocardial cell necrosis and dystrophic calcification; 4+, extensive inflammatory infiltration, necrosis, and dystrophic calcification. Mean values $\pm$ SD ($n = 5$). Means with different letters differ significantly at the $p < 0.05$ level.

Table 2
Effect of Fe and vitamin E status on cardiac viral titers of mice infected with CVB3/0

Diet		Cardiac viral titers (log $TCID_{50}$/g)				
Fe (ppm)	Vit E (ppm)	Day 3	Day 5	Day 10	Day 14	Day 21
35	100	0.7 ± 0.9^a	5.7 ± 0.5^a	2.6 ± 1.0^a	ND	ND
35	0	1.9 ± 0.8^a	5.8 ± 0.4^a	5.0 ± 0.4^b	1.4 ± 0.8^a	ND
1050	100	$4.1 \pm 0.4^{a,b}$	5.8 ± 0.5^a	6.0 ± 0.2^b	1.1 ± 0.3^a	ND
1050	0	5.9 ± 0.3^b	8.5 ± 0.4^b	7.5 ± 0.4^c	1.8 ± 0.9^a	ND

Mean values $\pm$ SD ($n = 5$). Means in the same column with different superscript letters differ significantly at the $p < 0.05$ level (ND, no detectable virus).

toward increasing TBARS with increased dietary iron in the incubated liver homogenates from vitamin E-supplemented infected mice.

Discussion

The results presented here provide yet one more example of how increased oxidative stress in the host can increase the virulence of the coxsackievirus. Previous work from our laboratories [6,7,18–21] has demonstrated the following:

1. Dietary deficiency of either vitamin E or selenium increases the heart muscle damage caused by a myocarditic strain of coxsackievirus.
2. Dietary deficiency of either vitamin E or selenium allows a benign amyocarditic strain of coxsackievirus to convert to a virulent myocarditic strain.
3. Feeding fish oil, a known vitamin E antagonist [8], intensifies the heart muscle damage caused by a myocarditic virus in vitamin E-deficient mice.
4. Feeding *N,N*′ -diphenyl-*para*-phenylenediamine, a synthetic antioxidant with potent vitamin E activity that has no structural similarity to tocopherol [22], prevents the increased heart muscle damage caused by a myocarditic strain of coxsackievirus in vitamin E-deficient mice.
5. Administration of inorganic mercury, a metabolic antagonist of selenium [23], allows a benign amyocarditic strain of coxsackievirus to cause heart damage and death in nondeficient mice.
6. A benign amyocarditic strain of coxsackievirus converts to a virulent myocarditic strain when inoculated into glutathione peroxidase-1 knockout mice [21].

Table 3
Effects of Fe and vitamin E status on hepatic TBARS in mice infected with CVB3/0

Diet		Hepatic TBARS (pmol/mg protein)
Fe (ppm)	Vitamin E (ppm)	
35	100	64 ± 2^a
35	0	88 ± 5^b
1050	100	$77 \pm 2^{a,b}$
1050	0	88 ± 5^b

Mean values $\pm$ SD ($n = 5$). Means with different superscript letters differ significantly at the $p < 0.05$ level.

All these observations, plus those reported here regarding iron overload, are consistent with our working hypothesis that increased oxidative stress in the host is responsible for the variety of effects on viral virulence seen due to the different experimental manipulations employed in our studies.

Several lines of evidence indicate that lipid peroxidation is increased during chronic iron overload, particularly in the liver, the organ that accumulates the most iron under conditions of overexposure. For example, the production of numerous by-products of lipid peroxidation (conjugated dienes, pentane, F2-isoprostanes, TBARS, etc.) is increased when animals are given excess iron [24–27]. Moreover, liver tocopherol levels decrease when very high levels of iron are fed [28]. In our experiment, we observed an increased production of TBARS in liver homogenates of our vitamin E-deficient mice. Under our conditions, hepatic vitamin E levels were somewhat lower in the vitamin E-supplemented group receiving the high level of dietary iron compared to the E-supplemented group fed the lower level of dietary iron.

The relevance of those oxidant-induced changes in the liver to the pathogenesis of a cardiotrophic virus such as coxsackievirus may not be obvious. However, this virus normally replicates in the liver during its infectious cycle before going to the heart (see Fig. 3 in Ref. [18]). Thus, the virus would be exposed to the strongly oxidizing environment of the vitamin E-deficient liver containing high levels of iron before reaching the heart. If the virus converts to virulence by virtue of a genomic change as seen with other instances of oxidative stress [29,30], perhaps the passage of the virus through the oxidatively stressed liver provides the opportunity for this conversion.

The peak cardiac viral titers were correlated with the extent of the observed heart muscle damage. The highest peak viral titer occurred in the vitamin E-deficient mice fed the high level of dietary iron and these mice also experienced the greatest degree of heart muscle damage. Interestingly, high viral titers were seen at day 3 post-infection in the high iron groups, compared with the groups fed a diet adequate in iron, whether or not vitamin E was added to the diet. Because the virus replicates in the liver before cardiac replication, the increased iron load in the liver may have provided an environment for accelerated viral replication. Clearly, the impact of oxidative stress in general and of iron status in particular on the severity of viral infection merits further attention.

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